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03-20-03

60456000 .A3 PROV

JC680 U.S. PTO 03/17/03

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PTO/SB/16 (8-00)
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J1040 U.S. PTO 60/456000 03/17/03

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TITLE OF THE INVENTION (280 characters max)

INHIBITORS OF ZINC METALLOPROTEINASES

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☒ Specification *Number of Pages* **23**

☒ Drawing(s) *Number of Sheets* **17**

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number

☐ Other (specify)

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Respectfully submitted,

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Docket Number: **P00252US0**

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Docket Number	P00252US0	Type a plus sign (+) inside this box →	+

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Invention: **INHIBITORS OF ZINC METALLOPROTEINASES**

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PROVISIONAL SPECIFICATION

for

LETTERS PATENT

on

INHIBITORS OF ZINC METALLOPROTEINASES

by

Darryl Rideout, Vladimir Tseitin, Mark Shenderovich
Ed Semple, Ruth Nutt, Venkatachalapathi Yalamoori and Chung-Ying Tsai

Number of drawing sheets: 17

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Description

Inhibitors of Zinc Metalloproteinases

Field of the Invention

The present invention relates to the prophylaxis and treatment of anthrax infections and, more particularly, to compounds that act as specific inhibitors of anthrax Lethal Factor (LF) activity, methods and means for making such inhibitors and their use as pharmaceuticals.

Background of the Invention

Anthrax is a zoonotic illness recognized since antiquity. In the 1870s, Robert Koch demonstrated for the first time the bacterial origin of a specific disease, with his studies on experimental anthrax, and also discovered the spore stage that allows persistence of the organism in the environment. Shortly afterward, *Bacillus anthracis* was recognized as the cause of woolsorter disease (inhalational anthrax). William Greenfield's successful immunization of livestock against anthrax soon followed in 1880, although Louis Pasteur's 1881 trial of a heat-cured anthrax vaccine in sheep is usually remembered as the initial use of a live vaccine.

Human cases of anthrax are invariably zoonotic in origin, with no convincing data to suggest that human-to-human transmission has ever taken place. Primary disease takes one of three forms: (1) Cutaneous, the most common, results from contact with an infected animal or animal products; (2) Inhalational is much less common and a result of spore deposition in the lungs, while (3) Gastrointestinal is due to ingestion of infected meat. Most literature cites cutaneous disease as constituting the large majority (up to 95%) of cases.

Bacillus anthracis is a large, gram-positive, sporulating rod, with square or concave ends. Growing readily on sheep blood agar, *B. anthracis* forms rough, gray-white colonies of four to five mm, with characteristic comma-shaped or "comet-tail" protrusions. Several tests are helpful in differentiating *B. anthracis* from other *Bacillus* species. *Bacillus anthracis is* characterized by an absence of the following: Hemolysis,

motility, growth on phenylethyl alcohol blood agar, gelatin hydrolysis, and salicin fermentation. *Bacillus anthracis* may also be identified by the API-20E and API-50CHB systems used in conjunction with the previously mentioned biochemical tests. Definitive identification is based on immunological demonstration of the production of protein toxin components and the poly-D-glutamic acid capsule, susceptibility to a specific bacteriophage, and virulence for mice and guinea pigs.

The virulence of *B anthracis* is dependent on two toxins, lethal toxin and edema toxin, as well as on the bacterial capsule. The importance of a toxin in pathogenesis was demonstrated in the early 1950s, when sterile plasma from anthrax-infected guinea pigs caused disease when injected into other animals (Smith, H. and J. Keppie, *Nature* **173**:869-870 (1954)). It has since been shown that the anthrax toxins are composed of three entities, which in concert lead to some of the clinical effects of anthrax (Stanley, J.L. and H. Smith, *J. Gen Microbiol* **26**:49-66 (1961); Beall, F.A. *et al.*, *J. Bacteriol* **83**:1274-1280 (1962)). The first of these, protective antigen, is an 83kd protein so named because it is the main protective constituent of anthrax vaccines. The protective antigen binds to target cell receptors and is then proteolytically cleaved of a 20kd fragment. A second binding domain is then exposed on the 63kd remnant, which combines with either edema factor, an 89kd protein, to form edema toxin, or lethal factor, a 90kd protein, to form lethal toxin (Leppla, S.H. *et al.*, *Salisbury Med Bull Suppl.*, **68**:41-43 (1990)). The respective toxins are then transported across the cell membrane, and the factors are released into the cytosol where they exert their effects. Edema factor, a calmodulin-dependent adenylate cyclase, acts by converting adenosine triphosphate to cyclic adenosine monophosphate. Intracellular cyclic adenosine monophosphate levels are thereby increased, leading to the edema characteristic of the disease (Leppla, S.H., *Proc Natl Acad Sci USA* **79**:3162-3166 (1982)). The action of lethal factor, believed to be a metalloprotease, is less well understood. Lethal toxin has been demonstrated to lyse macrophages at high concentration, while inducing the release of tumor necrosis factor and interleukin 1 at lower concentrations (Hanna, P.C. *et al.*, *Proc Natl Acad Sci USA* **90**:10198-10201 (1993); Freidlander, A.M., *J Biol Chem.* **261**:7123-7126 (1986)).

It has been shown that a combination of antibodies to interleukin 1 and tumor necrosis factor was protective against a lethal challenge of anthrax toxin in mice, as was the human interleukin 1 receptor antagonist (Hanna, P.C. *et al.*, *Proc Natl Acad Sci USA* 90:10198-10201 (1993)). Macrophage-depleted mice were shown to resist lethal toxin challenge, but to succumb when macrophages were reconstituted. The genes for both the toxin and the capsule are carried by plasmids, designated pXO1 [33] and pX02, respectively (Green, B.D. *et al.*, *Bacillus anthracis Infect Immunol.* **49**:291-297 (1985); Uchida, I. *et al.*, *J Gen Microbiol.* **131**:363-367 (1985)).

Disease occurs when spores enter the body, germinate to the bacillary form, and multiply. In cutaneous disease, spores gain entry through cuts, abrasions, or in some cases through certain species of biting flies. Germination is thought to take place in macrophages, and toxin release results in edema and tissue necrosis but little or no purulence, probably because of inhibitory effects of the toxins on leukocytes. Generally, cutaneous disease remains localized, although if untreated it may become systemic in up to 20% of cases, with dissemination via the lymphatic system. In the gastrointestinal form, *B. anthracis is* ingested in spore-contaminated meat, and may invade anywhere in the gastrointestinal tract. Transport to mesenteric or other regional lymph nodes and replication occur, resulting in dissemination, bacteremia, and a high mortality rate. As in other forms of anthrax, involved nodes show an impressive degree of hemorrhage and necrosis.

The pathogenesis of inhalational anthrax is more fully studied and understood. Inhaled spores are ingested by pulmonary macrophages and carried to hilar and mediastinal lymph nodes, where they germinate and multiply, elaborating toxins and overwhelming the clearance ability of the regional nodes. Bacteremia occurs, and death soon follows.

Penicillin remains the drug of choice for treatment of susceptible strains of anthrax, with ciprofloxacin and doxycycline employed as suitable alternatives. Some data in experimental models of infection suggest that the addition of streptomycin to penicillin may also be helpful. Penicillin resistance remains extremely rare in naturally occurring strains; however, the possibility of resistance should be suspected in a

biological warfare attack. Cutaneous anthrax may be treated orally, while gastrointestinal or inhalational disease ordinarily should receive high doses of intravenous antibiotics (penicillin G, 4 million units every 4 hours; ciprofloxacin, 400 mg every 12 hours; or doxycycline hyclate, 100 mg every 12 hours). The more severe forms require intensive supportive care and have a high mortality rate despite optimal therapy. The use of anti-anthrax serum, while no longer available for human use except in the former Soviet Union, was thought to be of some use in the pre-antibiotic era, although no controlled studies were performed.

Although anthrax vaccination dates to the early studies of Greenfield and Pasteur, the "modern" era of anthrax vaccine development began with a toxin-producing, unencapsulated (attenuated) strain in the 1930s. Administered to livestock as a single dose with a yearly booster, the vaccine was highly immunogenic and well tolerated in most species, although somewhat virulent in goats and llamas. This preparation is essentially the same as that administered to livestock around the world today. The first human vaccine was developed in the 1940s from nonencapsulated strains. This live spore vaccine, similar to Sterne's product, is administered by scarification with a yearly booster. Studies show a reduced risk of 5-to-15-fold in occupationally exposed workers (Shlyakhov, E.N and E. Rubenstein, *Vaccine* 12:727-730 (1994)).

British and U.S. vaccines were developed in the 1950s and early 1960s, with the U.S. product an aluminum hydroxide-adsorbed, cell-free culture filtrate of an unencapsulated strain (V770-NP1-R), and the British vaccine an alum-precipitated, cell-free filtrate of a Sterne strain culture. The U.S. vaccine has been shown to induce high levels of antibody only to protective antigen, while the British vaccine induces lower levels of antibody to protective antigen but measurable antibodies against lethal factor and edema factor (Turnbull, P.C.B. *et al.*, *Infect Immunol.* 52:356-363 (1986); Turnbull, P.C.B. *et al.*, *Med Microbiol Immunol.* 177:293-303 (1988)). Neither vaccine has been examined in a human clinical efficacy trial. A high number of the recipients of the vaccine have reported some type of reaction to vaccination. The preponderance of these events was minor. Manufacturer labeling for the current Michigan Department of Public Health anthrax vaccine adsorbed (AVA) product cites a 30% rate of mild local reactions

and a 4% rate of moderate local reactions with a second dose. The current complex dosing schedule for the AVA vaccine consists of 0.5mL administered subcutaneously at 0, 2, and 4 weeks, and 6, 12, and 18 months, followed by yearly boosters.

Animal studies examining the efficacy of available anthrax vaccines against aerosolized exposure have been performed. While some guinea pig studies question vaccine efficacy, primate studies have support its role. In recent work, rhesus monkeys immunized with 2 doses of the AVA vaccine were challenged with lethal doses of aerosolized B *anthracis* spores. All monkeys in the control group died 3 to 5 days after exposure, while the vaccinated monkeys were protected up to 2 years after immunization (Ivins, B.E. *et al.*, *Salisbury Med Bull Suppl.* **87**:125-126 (1996)). Another trial used the AVA vaccine in a 2-dose series with a slightly different dosing interval, and again found it to be protective in all rhesus monkeys exposed to lethal aerosol challenge (Pitt, M.L.M. *et al.*, *Salisbury Med Bull Suppl.* **87**:130 (1996)). Thus, available evidence suggests that two doses of the current AVA vaccine should be efficacious against an aerosol exposure to anthrax spores. In addition, a highly purified, minimally reactogenic, recombinant protective antigen vaccine has been investigated, using aluminum as well as other adjuvants. Other approaches include cloning the protective antigen gene into a variety of bacteria and viruses, and the development of mutant, avirulent strains of *B anthracis*. One significant limitation on the use of vaccines is that existing vaccines provide no protection against a number of strains of *B. anthracis*.

Recent incidents, such as the suspected use of biological and chemical weapons during the Persian Gulf War, underscore the threat of biological warfare either on the battlefield or by terrorists. Anthrax has been the focus of much attention as a potential biological warfare agent for at least six decades, and modeling studies have shown the potential for use in an offensive capacity. Dispersal experiments with the simulant *Bacillus globigii* in the New York subway system in the 1960s suggested that release of a similar amount of *B. anthracis* during rush hour would result in 10,000 deaths. On a larger scale, the World Health Organization estimated that 50kg of *B anthracis* released upwind of a population center of 500,000 would result in up to 95,000 fatalities, with an additional 125,000 persons incapacitated (Huxsoll, D.L. *et al.*, *JAMA* **262**:677-679

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(1989)). Both on the battlefield and in a terrorist strike, *B. anthracis* has the attribute of being potentially undetectable until large numbers of seriously ill individuals present with characteristic signs and symptoms of inhalational anthrax.

Given these findings, efforts to prevent the disease or to ameliorate or treat its effects are of obvious importance. The U.S. military's current M17 and M40 gas masks provide excellent protection against the 1 to 5μm particulates needed for a successful aerosol attack. Assuming a correct fit, these masks would be highly effective if in use at the time of exposure. Some protection might also be afforded by various forms of shelter. The pre-exposure use of the current AVA anthrax vaccine, which is approved by the U.S. Food and Drug Administration, appears to be an important adjunct. Results of primate studies also support the concept of post-exposure antibiotic prophylaxis. One study showed that 7 of 10 monkeys given penicillin, 8 of 9 given ciprofloxacin, 9 of 10 treated with doxycycline, and all 9 receiving doxycycline plus post-exposure vaccination survived a lethal aerosol challenge, with all animals receiving antibiotics for 30 days following exposure (Friedlander, A.M. *et al.*, *J Infect Dis.* **167**:1239-1242 (1993). Earlier research suggested that short courses of prophylactic antibiotics delayed but did not prevent clinical disease (Henderson, D.W. *et al.*, *J Hyg.* **54**:28-36 (1956). Accordingly, in the event of documented exposure, prolonged prophylactic antibiotic use, as well as vaccination, would be mandatory. In the biological warfare setting, the differential diagnosis of inhalational anthrax would include plague and tularemia. Fluoroquinolones also have activity against these diseases, supporting the use of ciprofloxacin and perhaps other drugs of this class as either a pre-exposure or post-exposure measure.

It is therefore apparent that while certain prophylactic and treatment schemes may prove useful in preventing or ameliorating anthrax infections, there remains a compelling need to improve the arsenal of techniques and agents available for this purpose.

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Detailed Description of the Invention

The present invention provides methods, compounds and compositions for treating anthrax infections by inhibiting anthrax lethal factor (LF) activity. The novel compositions for use herein are LF inhibitors. These substances function by binding to the LF cleavage site, and preventing the LF from catalyzing its physiological substrate. LF inhibitors are useful, either alone or together with other therapeutic compositions, in the prevention and treatment of anthrax infections. Although the term "infection" is ordinarily used in its epidemiological sense, it will readily be recognized that "infections" by *Bacillus anthracis* spp., or invasions by LF, can occur naturally or be purposefully induced.

Anthrax toxin, produced by *Bacillus anthracis*, is composed of three proteins: Protective antigen (PA), edema factor (EF) and LF. Protective antigen is an 83kd protein that binds to specific cell surface receptors and is then proteolytically activated to a 63kd fragment (PA63), which forms a membrane channel that mediates entry of EF or LF into the cell. PA combines with either EF, an 89kd protein, to form edema toxin, or LF, a 90kd protein, to form lethal toxin (Leppla, S.H. *et al.*, *Salisbury Med Bull Suppl.*, **68**:41-43 (1990)). The respective toxins are then transported across the cell membrane, and the factors are released into the cytosol where they exert their effects. EF, a calmodulin-dependent adenylate cyclase, acts by converting adenosine triphosphate to cyclic adenosine monophosphate. Intracellular cyclic adenosine monophosphate levels are thereby increased, leading to the edema characteristic of the disease (Leppla, S.H., *Proc Natl Acad Sci USA* **79**:3162-3166 (1982)).

The action of LF, the dominant virulence factor produced by *Bacillus anthracis*, and believed to be a metalloprotease, is less well understood. Lethal toxin has been demonstrated at high concentration to lyse macrophages, while inducing the release of tumor necrosis factor and interleukin 1 at lower concentrations (Hanna, P.C. *et al.*, *Proc Natl Acad Sci USA* **90**:10198-10201 (1993); Freidlander, A.M., *J Biol Chem.* **261**:7123-7126 (1986)). LF is a 776 amino acid protein that contains a putative zinc-binding site (HEFGF) at residues 686-690, a characteristic of metalloproteases. Mutation of the H or E residues is reported to inactivate LF, and reduces its zinc-binding activity.

One useful approach to providing agents, which will serve as inhibitors of LF activity, is to model the protein surface structure of MAP kinase kinase 1 (MAPKK1), a physiological substrate cleaved by LF. In conjunction, the consensus structural features of MAPKK1 and MAPKK2 that contain the LF cleavage site will provide a basis for designing non-peptide inhibitors of LF activity.

Molecular Design Considerations

The docking models of the MAPKK1 fragment and the In-2-LF inhibitor are used for the improvement of existing small molecule inhibitors and the de-novo design of new inhibitors. The resulting MD trajectory of the LF-MAPKK1 fragment complex is currently being used to provide the basis for the design of an improved *DynaPharm*® pharmacophore template, which is a central part of a virtual library screening strategy for discovery and optimization of more potent inhibitors.

First, flexible and rigid regions on the surfaces of LF and MAPKK1 in the cleavage region of the complex model are being determined from the MD trajectory. Detailed analyses are being carried out at the surface of the N-terminal portion of MAPPK1 residing in the LF active site in order to extract characteristics of the interacting residues over the trajectory. Residues at the interface are identified and grouped using the following criteria: 1) contribution to the energetics of its binding to LF and 2) analysis of hydrophobicity. After grouping residues, the distances and angles between each residue in the group are measured and tabulated. Whenever aromatic or non-aromatic rings are involved, the centers of the rings are used for distance evaluation. For side chains longer than Alanine, the center of mass of the residue are used as the reference point for measuring the distances and angles. This will yield the desired virtual constructs of the residues (including dynamic motion) for constructing a *DynaPharm*® template and for more refined docking-based approaches.

The new docking models have been applied to a 1-hydroxyhydropyrazin-2-one scaffold identified previously. Computational docking studies on hydroxypyrazinones using the LF structure suggested that the ring hydroxamic acid group would be prevented from chelating zinc because of unfavorable steric interactions between ligand and protein.

[redacted]

However, these studies also suggested that derivatives of these structures tethered to other zinc-binding groups (such as carboxylic acid or thiol) could show activity. Figure C-3 shows a few examples of hydroxypyrazinones exhibiting activity in the Western Blot assay. Only structure SBI-031592, which contains an additional carboxylic acid moiety, showed activity in the FRET assay. The methyl ester of SBI-031592 was inactive in this assay, suggesting that the carboxylic acid moiety in itself is important for activity, while the hydroxamic acid groups in the pyrazinone ring are insignificant. Analogs of hydroxypyrazinones without hydroxyl groups (pyrazinones and alkoxypyrazinones) did not differ significantly from hydroxypyrazinones in the Western Blot assay, a result that is also inconsistent with a model involving zinc binding to the ring hydroxamic acid group in these compounds.

In light of these SAR results for hydroxypyrazinones and the computational predictions, attention was given to other scaffolds, including hydroxamic acids, carboxylic acids, thiols and barbituric acids. Computational docking studies and similarity searching helped to identify scaffolds related to SBI-031592, containing nitrogen heterocycles linked to 2 or 3 phenyl rings, and exemplified by scaffolds B, E, F, G, H, J and K in Figure C-4. Computational studies based on interactions of the MAPKK1 peptide with LF and further similarity searching helped to identify scaffolds A, C, and D. Based on both computational and assay data, scaffolds C and J in Figure C-4 were identified as of particular interest. Preliminary results suggest that some inhibitors with scaffold J exhibit selectivity against LF versus MMP-1 (IC_{50} (MMP-1)/ IC_{50} (LF) >6). Scaffold C is particularly interesting because of its relative potency and drug-like nature. The drug-like nature of scaffold C derives from the fact that one molecule in this class has been tested in mice as a candidate inhibitor of another target (TNF sheddase) and successfully prevented the lethal effects of lipopolysaccharide + galactosamine by blocking TNF synthesis (Mohler, K.M. *et al., Nature* 370:218 (1994). The compound thus appears to be non-toxic in mice and sufficiently bioavailable and stable to reach the target enzyme. Derivatives of scaffold C will be examined in an effort to improve its potency.

[redacted]

Structures and measured IC_{50} values (FRET assay) for some of the hydroxamic acid derivatives are presented in Figure C-5. Note that three of the derivatives have single digit micromolar IC_{50} values and one has an IC_{50} value of 1.6μM. While more analog compounds are still being designed, synthesized and tested to gain a better understanding of structure/activity relationships, some preliminary conclusions can nevertheless be made. Scaffold B is an inhibitor, while its enantiomer is inactive, consistent with a specific interaction with LF near the binding site as opposed to nonspecific protein binding. For scaffold C, the most promising to date, the 2 fused aromatic rings (naphthyl and indole) and the alkyl group on the succinic diamide appear to contribute significantly to binding, while R3 is less critical. Intermediates for the synthesis of more than 40 other members of the C scaffold family have recently been prepared, using the docking model depicted in Figure C-6 as a guide.

Structures and measured IC_{50} values for some of the carboxylic acid derivatives examined to date are presented in Figure C-7. Three of these derivatives are active in the single digit μM range. For the carboxylic acid scaffold series G, although there is little dependence on chain length, n = 5 appears to be close to optimal. Competitive inhibition has been observed for individual compounds in the G and J scaffolds, consistent with binding near the active site. A docking model of a member of the J series bound to the LF active site is depicted in Figure C-6. Note that direct binding to zinc occurs for the hydroxamic acid moiety or the carboxylate moiety in both docking models shown in Figure C-6.

The X-ray crystal structure of LF has been computationally refined and combined with the AHM model of MAPKK1 to derive a full solvated MD trajectory of a bound LF-MAPKK2 fragment complex, and a bound complex of LF and the inhibitor In-2-LF. The bound complex models have been used to design and screen new scaffolds and derivatives of candidate LF inhibitors. Compounds with IC_{50} activities in the single digit micromolar range have been discovered for 3 novel scaffold families, with the most active to date being 1.6μM.

At that point where 100nM range compounds are identified, the co-crystallization task will be initiated for the most promising compounds, which will provide even more

accurate structural information with which to further optimize the LF inhibitor candidates toward the 1 – 10nM activity goal.

Strategies for improving stability to enzymatic degradation will include amide replacement by C-C bond containing moieties or heterocycles, replacements of readily oxidized sites (e.g. replacement of phenyl by 4-fluorophenyl, 1-butyl by 2-fluoro-1-butyl). Strategies to minimize toxicity will include replacement of potentially toxophoric groups by less toxic bioisosteres (e.g. replace 3-nitrophenyl with 3-aminosulfonylphenyl or 3-acetylphenyl) and changes that improve selectivity for LF versus other metalloenzymes. Strategies to maximize selectivity against LF compared to other enzymes will include computationally guided alterations in the size of appropriate moieties. Using this same approach, extension of methyl groups to heptyl or benzhydryl has been used to increase the selectivity of certain hydroxamic acids between matrix metalloprotease subtypes by >500 fold (Whittaker *et al.*, *Chem. Rev.* V. 99, 2735-2776 (1999); Miller *et al.*, *Bioorg. Med. Chem Lett* 7:193 (1997)). Strategies for improving bioavailability will include enhancing solubility by decreasing symmetry, introducing branching, reducing molecular weight, and substituting hydrophobic groups with polar groups such as alkoxy and aliphatic amines. Both solubility and membrane permeability are enhanced as needed by making substitutions that optimize log P and log D values, such as replacing arginine side chains with less polar 2-aminopyridines, replace amide CONH with $COCH_2$, thiazole, oxadiazole, oxazole, alkene, etc. Figure D-4 shows examples of how some of these strategies are applied, using the LF inhibitor scaffolds C and J as starting points. The structures of those more promising inhibitors are treated similarly as for scaffolds C and J in Figure D-4 using similar types of structural alterations and bioisosteric replacements.

Prodrug strategies are applied to agents that are predicted to show poor oral bioavailability, but are otherwise promising in terms of ADMET properties and potency when administered subcutaneously (s.c.) to mice. These will include strategies that have proven useful for other metalloproteinase inhibitors, e.g. ethyl esters as prodrugs of carboxylic acids and thioethers as prodrugs of thiols (Alton *et al.*, *J. Chromatogr*

579:307-317 (1992); Noble *et al.*, *J Pharmacol Exp Ther* **261**:181-90 (1992); Skiles *et al.*, *Current Medicinal Chemistry* **8**:425-474 (2001)).

The basis for the substitutions proposed in analogs depicted in Figure D-4 is as follows (small letter designations in the list correspond to the letters in Figure D-4):

a) CH >> CF to improve metabolic stability.

b) Increased steric bulk within cavity to improve selectivity against LF versus other metalloenzymes.

c) CH >>N to optimize log D for bioavailability.

d) Decrease rotatable bonds through structural constraints for improved bioavailability.

e) α-alkylation enhances hydroxamic acid metabolic stability.

f) H>>CF_3 to adjust log D for bioavailability, and to improve metabolic stability.

g) Replace amide with heterocycle for improved metabolic stability, optimization of log D for bioavailability, decrease in NH bond count (Lipinski *et al.*, *Adv. Drug Del. Rev.* **23**:3-25 (1997)).

h) Replace amide with C-C for improved metabolic stability, optimize log D for bioavailability, decrease in NH bond count (Lipinski's rules). One example of significant improvement in oral bioavailability of a metalloprotease inhibitor through replacement of NH with CH_2 has been described by Chapman *et al.* *Bioorg. Med. Chem. Lett.* **6**:803 (1996) (Lipinski *et al.*, 1997).

j) Replace with alternate heterocycle for improved solubility and drug-like character.

k) CF_3>>OCH_3 for improved solubility, optimization of log D for bioavailability.

m) Eliminate phenyl group for improved solubility, optimization of log D for bioavailability, and lower molecular weight.

n) CF_3>>F for lower molecular weight.

p) Replace hydroxamic acid moiety with thiol for decreased mutagenicity; thioester prodrug for increased bioavailability.

q) Create acetoxymethyl ester of carboxylic acid as prodrug to improve oral bioavailability.

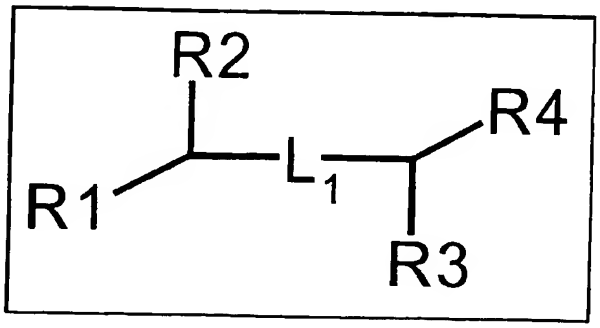
r) Append tertiary amine for improved solubility.

Metalloproteinase Inhibitor Compounds of the Invention

According to the design considerations and strategies described above, the compounds according to the following structural formula will find use as metalloproteinase inhibitor compounds useful for treating anthrax infections by inhibiting

[redacted]

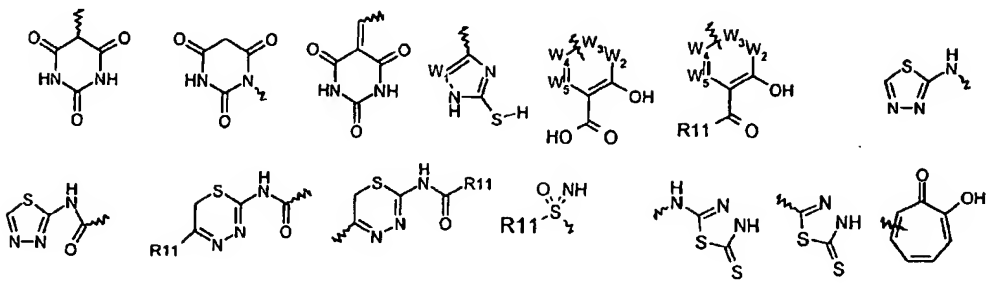
anthrax lethal factor (LF) activity. LF inhibitors are useful, either alone or together with other therapeutic compositions, in the prevention and treatment of anthrax infections, whether resulting from infection by *Bacillus anthracis* spp., or purposefully induced invasions by LF.



In the structural formula presented above,

R1 can be $(CHR5)_nY$ where n is 0 to 4, and Y is a group known to bind to zinc, including CONHOH, COOH, SH, ArSH, 2-hydroxybenzoate (linked at the 3,4,5, or 6-position), $NHCO(CHR11)_mSH$ (where m is 1 to 4), $PO(OH)_2$, PO(R11)OH, $SO_2NR11OH$ or NH(OH)COR11, and R5 and R11 are, independently, H, CH_3, amino, hydroxy, alkoxy, alkylthio, alkyl (C2-C10), branched alkyl (C3-C10), alkylthio, arylthio, alkylamino, amino, arylamino, aryl, heteroaryl, arylalkyl, heteroarylalkyl, arylalkenyl, heteroarylalkenyl, arylalkynyl, or heteroarylalkynyl. R1 is optionally further substituted with one or more of the following: NH_2, OH, halogen, alkyl, $CONH_2$, CONHOH, $C(NH)NH_2$, C(NH)NHOH, $NHC(NH)NH_2$, CN, NO_2, NR6R7 where R6 and R7 are H or alkyl and optionally form a ring. R5 can optionally form a ring with R2 or with R11.

Additional structures for Y are as follows:



Where W1-W5 are each independently CH, N, C-alkyl, C-OH, CF, CCl, CCF_3, $COCF_3$, $COCH_3$, or CBr.

R2 can be H, isobutyl, n-butyl, pentyl, alkyl (C1-C10), branched alkyl (C3-C10), cycloalkyl, cycloalkylmethyl (C3-C9 cycle), $Ar(CH_2)n$ (n is 0 to 4, Ar is phenyl, aryl, heteroaryl), arylalkenyl, heteroarylalkenyl, arylalkynyl, heteroarylalkynyl, alkenyl (C2-C8), alkynyl (C2-C8), cycloalkenyl (C4-C10), alkylthio, arylthio, alkylamino, arylamino.

R2 can optionally form a ring with R5, R11, L1, or R3. R2, R5 and R11 are optionally substituted with one or more of the following: NH_2, OH, halogen, alkyl, CF_3, CF_3O, CF_3S, alkoxy, alkylthio, $CONH_2$, CONHOH, $C(NH)NH_2$, CN, NO_2, C(NH)NHOH, $NHC(NH)NH_2$, NR6R7 where R6 and R7 are H or alkyl and optionally form a ring.

R3 can be H, isobutyl, n-butyl, n-pentyl, cyclobutylmethyl, phenethyl, alkyl (C1-C10), branched alkyl (C1-C10), phenyl substituted with aryl or heteroaryl at the 2-, 3-, or 4-positions, aryl, heteroaryl (including thiophenyl), -R8Ar where Ar is 1-napthyl, 2-napthyl, 4-phenylphenyl, phenyl, 3-phenylphenyl, indolyl, benzthiophenyl, aryl or heteroaryl and R8 is a linker chosen from the following, in both orientations: bond, CH_2, $(CH_2)_2$, CH_2NHCH_2, $CH_2CH_2CONHCH_2$, $CH_2CH_2CONHCH_2CH_2$, $CH_2CH_2NHCH_2$, $CH_2CH_2CH_2NHCH_2$, $CH_2NHCH_2CH_2$, $(CH_2)q$ where q is 3 to 7, (CHR9)r where r is 1 to 7 and R9 is independently H, alkyl (C1-C10), branched alkyl (C3-C10), cycloalkyl (C3-

C10), cycloalkylalkyl (C4-C14), alkyl thio, amino, alkyl amino, dialkylamino, (CHR9)sX(CHR9)t where s + t is 2 to 8, X is O, S, SO, SO_2, NH, CONH, NHCO, SO_2NH, $NHSO_2$ or NR9 and R9 is independently H, alkyl (C1-C10), branched alkyl (C3-C10), cycloalkyl (C3-C10), cycloalkylalkyl (C4-C14), acyl, alkyl thio, amino, alkyl amino, or dialkylamino. Carbon-carbon single bonds in R8 can optionally be substituted with double or triple bonds. R3 can optionally form a ring with R2, L1, or R4. R3 is optionally substituted with one or more of the following NH_2, OH, halogen, alkyl, CF_3, CF_3O, CF_3S, alkoxy, alkylthio, $CONH_2$, CONHOH, $C(NH)NH_2$, CN, NO_2, C(NH)NHOH, $NHC(NH)NH_2$, NR6R7 where R6 and R7 are H or alkyl and optionally form a ring.

R4 can be H, alkyl (C1-C10), branched alkyl (C1-C10), arylalkyl, heteroarylalkyl, CONHR10 where R10 is H, methyl, alkyl (C2-C10), branched alkyl (C3-C10), benzyl, phenethyl, arylalkyl, heteroarylalkyl, CHR11CONHR12 (where R11 is H, ethyl, methyl, isobutyl, sec-butyl, phenyl, benzyl, phenethyl, indolylmethyl, aminoalkyl, hydroxyalkyl, alkyl (C1-C10), branched alkyl (C3-C10), cycloalkyl (C3-C10), aryl, heteroaryl, arylalkyl, heteroarylalkyl; and where R12 is H, alkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, aminoalkyl, hydroxyalkyl). R4 can optionally form a ring with L1 or R3. R4 is optionally substituted with one or more of the following: NH_2, OH, halogen, alkyl, CF_3, CF_3O, CF_3S, alkoxy, alkylthio, $CONH_2$, CN, NO_2, CONHOH, $C(NH)NH_2$, C(NH)NHOH, $NHC(NH)NH_2$, NR6R7 where R6 and R7 are H or alkyl and optionally form a ring.

L1 can be CONH, NHCO, CH_2NH, $NHCH_2$, CH=CH, $C(NH_2)$=N, N=$C(NH_2)$, arylene (linked 1,2-; 1,3-; or 1,4), heteroarylene (linked 1,2-; 1,3-; or 1,4), ethynyl, CH=CF, CF=CH, CF=CF, CH_2CH_2, $C(CH_3)$=CH, CH=$C(CH_3)$, SO_2NH, SO_2, $COCH_2$, CH_2CO, $CNOHCH_2$, CH_2CNOH, $C(CF_3)$=CH, CH=$C(CF_3)$, SO_2CH_2, CH_2SO_2, $SOCH_2$, CH_2SO, CH_2CHOH, $CHOHCH_2$, lower cycloalkyl (C3-C6), or CHOHCHOH. L1 is optionally substituted with one or more of the following: NH_2, OH, halogen, alkyl, CF_3, CF_3O,

CF_3S, alkoxy, alkylthio, $CONH_2$, CONHOH, $C(NH)NH_2$, C(NH)NHOH, NHC(NH)NH2, NR6R7 where R6 and R7 are H or alkyl and optionally form a ring.

For all chiral centers on the scaffold, in the linker L1, and in substituents R1 through R4, both R and S stereochemistry are contemplated. For all double bonds in the linker L1, and in substituents R1 through R4, both E and Z stereochemistry are included.

As used herein: "Aryl" includes phenyl, naphthyl, phenanthrenyl, anthracenyl, biphenyl, terphenyl, phenylnaphthyl and azulenyl linked from any position. "Heteroaryl" is any monocyclic, fused bicyclic or fused tricyclic aromatic system for which at least one ring atom is O, N, or S, including thiophene, pyrrole, noxazole, furan, thiazole, imidazole, pyrazole, isoxazole, isothiazole, oxadiazole, triazole, tetrazole, thidiazole, pyridazine, pyrimidine, pyrazine, thiadiazole, triazine, indolizine, indole, benzofuran, benzothiophene, benzimidazole, benzthiazole, purinc, quinoline, isoquinoline, cinnoline, phtalazine, quinazoline, naphthyridine, pteridine, carbazole, acridine, phenazine, dibenzofuran, dibenzothiophene, isomers of these, and fused aromatic ring systems (up to 3 rings) containing these, heteroaryl-aryls (up to 4 rings), aryl-heteroaryls (up to 4 rings) and heteroaryl-heteroaryls (up to 4 rings) attached from any position. Examples of heteroaryl-aryls: thienylphenyl, pyridylnaphthyl. Examples of aryl heteroaryls: biphenylthiazolyl, napthyl pyrmidinyl.

Aromatic and heteroaromatic rings can be optionally and independently further substituted with one to four of the following groups: R13, R13O, R13S, R13CO, R13OCO, R13SO, $R13SO_2$, $R13SO_2NH$, $R13NHSO_2$ in which R13 is H, aryl, heteroaryl, NH_2, OH, halogen, alkyl (C1-C10), heterocycloalkyl, heterocycloalkenyl, branched alkyl (C3-C8), cycloalkyl (C3-C8), bicycloalkyl (C4-C12), cycloalkenyl (C4-C9), bicycloalkenyl (C6-C12), arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, alkenyl, alkynyl, $CONH_2$, CONHOH, $C(NH)NH_2$, C(NH)NHOH, $NHC(NH)NH_2$, CN, NO_2, CF_3, OCF_3, SCF_3, CH_2CF_3, CH_3, perfluorinated alkyl (C1-C5), perfluorinated branched alkyl (C3-C5), perfluorinated cyclic alkyl (C3-C5), alkyl (C1-C10), alkoxy (C1-C10), alkylthio (C1-C9),arylthio, heteroarylthio, arylalkylthio, 2'-hydroxyethoxy, alkoxycarbonylmethoxy (C1-C4), dialkylamino (C1-C4

where the 2 alkyls optionally form a heteroalicyclic ring), difluoromethoxy, guanidine, guanidinoalkyl (C1-C5), $H_2N(NH)C(CH_2)_h$ where h is 0 to 6, $H_2N(NH)CNHO(CH_2)_j$ where j is 0 to 6, (2-pyridyl)amino, (2-pyridyl)aminoalkyl (C1-C6), perfluoroalkyl (C1-C4), perfluoroalkylthio (C1-C4), perfluoroalkoxy (C1-C4), 2-carboxyvinyl, alkanoyl (C1-C5), alkoxycarbonyl (C1-C4), or alkanoylamino (C1-C8). R13 may also be CONR7R7 or NR6R7 or SO_2NR6R7 or NR6COR7 or NR6SO2R7 where R6 and R7 are, independently, H, alkyl (C1-C10), branched alkyl (C3-C8), cycloalkyl (C3-C8), aryl, arylalkyl, arylalkenyl, arylalkynyl, alkenyl, alkynyl, heteroarylalkyl, hctcroarylalkenyl, heteroarylalkynyl, and where R6 and R7 optionally form a ring.

Specific examples of compounds of the above-identified structural formula which are within the scope of the present invention are provided in the attached Figure 1, together with preliminary assay results on their *in vitro* biological activity.

Synthesis of Inhibitor Compounds of the Invention

In general, the compounds of the present invention can be prepared in accordance with chemical synthetic protocols well known to those of skill in this art. One desirable category of such techniques is known by the generic term "combinatorial chemistry." Such techniques are well know in the art, and can be generally summarized as follows: For example, preparation of libraries can be by the "split synthesis" method, as described in Gallop *et al.*, *J. Med. Chem.*, **37**:1233-1251 (1994). The split synthesis procedure involves dividing a resin support into n equal fractions, in a separate reaction carry out a single reaction to each aliquot, and then thoroughly mixing all the resin particles together. Repeating the protocol for a total of x cycles can produce a stochastic collection of up to n^x different compounds. An alternative format is by preparing sub-libraries in the $O_3O_2X_1$ format, wherein two positions on the compounds, O_3 and O_2 are explicitly defined and a third position, X_1, varies. Such sub-libraries can be conveniently prepared by the tea-bag techniquc, as is known in the art, and described, for example in U.S. Pat. No. 4,631,211 and Houghten *et al.*, *Proc. Natl. Acad. Sci.*, **82**:5131-5135 (1985).

Alternatively, or in addition thereto, the iterative selection and enhancement process of screening and sub-library resynthesis can be employed. For example, a sub-

library of various R1 substituents can be screened to select the most active R1 substituent. The compound having the most active R1 is then resynthesized and with the R1 position being defined, a new R2 position mixture library is prepared, screened, and the most active R2 selected. The above process can then be repeated to identify the most active R substituents on the backbone structure.

In yet another approach, the positional scanning technique, only a single position is defined in a given sub-library and the most preferred substituent at each position of the compound is identified.

The advantage of synthetic combinatorial libraries (SCLs) made up of mixtures of tens of millions of different compounds is that they can be used to rapidly identify individual, active compounds without the need to individually synthesize, purify, and test every single compound. Since the libraries are in solution (i.e., not attached to a bead, pin, phage, glass, etc.) they can be screened in virtually any assay system.

Solution phase combinatorial chemistry methods can be used when the product can be separated from side products and starting materials through rapid techniques. Examples of these are: (1) selective precipitation of product and removal of byproducts and precursors by washing, (2) selective removal of side products and starting materials using chemically reactive polymers and/or ion exchange polymers ("scavenge"), (3) selective binding of product to a chemically reactive polymer, followed by removal of the product through a second chemical reaction ("capture") (4) selective binding of product to an ion exchange polymer, followed by removal with acid, base, or high salt buffer ("capture"), and (5) selective solubilization of product. Solution phase combinatorial chemistry approaches are covered in a recent set of reviews (*Tetrahedron*, **54**:3955-4150 (1998)).

The synthetic approaches can be optimally carried out using solution phase combinatorial chemistry. Several reactions are carried out simultaneously using a multiple reaction vessel block such as, but not limited to, the Charybdis Calypso™ temperature controlled blocks, with gas manifolds to maintain an argon or nitrogen atmosphere. Alternately, the reactions can be carried out simultaneously in multiple vials filled with argon or nitrogen and fitted with magnetic stirbars and

polytetrafluoroethylene-lined, sealed caps, by heating and stirring them simultaneously in a magnetic stirrer/heater such as, but not limited to, the Pierce ReactTherm™ III Heating/Stirring Module. The products are isolated by addition of water and filtration using a system such as, but not limited to, the Charybdis Calypso™ filtration block or polypropylene syringes fitted with filter disks made from polyethylene, polytetrafluoroethylene, or glass and attached to a vacuum manifold.

Representative synthetic schemes for some of the structures proposed in Figure D-4 are depicted in Figure D-5. Representative combinatorial libraries and their synthetic schemes are shown in Figure D-6. Individual compounds are synthesized using high-throughput methods and screened to determine synthetic feasibility and the activity of a representative structure. (High throughput procedures will include solid phase chemistry and solution phase chemistry with solid-phase reagents and scavengers. Where appropriate, microwave chemistry using Personal Chemistry Synthesizer instrumentation is carried out to increase the efficiency of library synthesis.) If the synthetic accessibility and potency are adequate, a virtual library (100-1000 structures) are constructed and added to the set of libraries to be used for the second round of *in silico* ADMET screening. After *in silico* screening has been used to remove structures that are unlikely to exhibit favorable ADMET profiles, the structures remaining in the virtual library are synthesized using high-throughput procedures.

Other in vitro testing:

Other *in vitro* studies will be desirable for full assessment of candidate LF inhibitors. These studies, listed here for the sake of completeness, are: (1) LF inhibitory activity in vitro (at SBI); (2) Efficacy against LT cytotoxicity in macrophages (external contract); (3) Ames test for mutagenicity (external contract - if the Ames test proves positive for compounds with otherwise favorable ADMET and activity profiles, more extensive genotoxicity and carcinogenicity studies in rats are carried out as needed); (4) set of PanLabs screens (external contract); and (5) cytotoxicity tests in human and rodent monocytic and hepatocyte cell lines (at SBI) (6) stability studies and (7) formulation.

[redacted]

Preclinical Toxicity And Efficacy Studies In Mice using LT Challenge

In this task, approximately 20 compounds are chosen, on the basis of the best overall performance in the set of *in vitro* ADMET screens in Task 2, for further evaluation in live animals using Lethal Toxin (LT) challenge (LT is the toxic combination of LF and the permeabilizing factor, PA). The goal is for this set of compounds to consist of at least 2 representatives of each structural subclass.

Mouse studies described in this section (acute toxicity studies and efficacy studies involving LT-injected mice) are carried out in female mice, A/J strain, 6 weeks of age, weighing approximately 20g each. The strain, gender and age were chosen based on a mean lifespan when exposed to 4 x LD50 of anthrax LT that is sufficiently long (mean 3.7 days) to allow the possibility of post-toxin treatment as well as prophylaxis (Welkos *et al., Infection and Immunity* **51**:795-800 (1986)). This 3.7-day lifespan is also similar to the mean lifespan (3 days) of mice infected with 5000cfu of *bacillus anthracis* spores. The planned trials, and associated schedules and protocols are presented in the following sub-sections.

Acute toxicity in mice

Single doses of drug candidates are injected s.c. into sets of 5 mice per dose level using 0.1, 0.3, 1, 3, and 10mg/kg. Animals are observed for 14 days to estimate the MTD or to determine the lower limit of the MTD (the highest dose at which no more than 10% of the mice show clear signs of toxicity). Mice are weighed daily and their food consumption measured. For this preliminary study, the signs of toxicity are limited to nausea, lethargy, anorexia, weight loss, abnormal fur texture, diarrhea or mortality within the 14-day observation period. Mice showing signs of pain due to toxic effects are euthanized immediately. Combination toxicity studies of each candidate at its MTD with ciprofloxacin will also be carried out, because compounds that exhibit significant adverse interactions with ciprofloxacin are not worthy of further consideration. Compounds must have maximum tolerated doses above 1mg s.c. for further consideration (the dose used for inhibition of the TACE metalloprotease by the compound). For mice

with an MTD > 1mg/kg, postmortem gross necropsy is carried out on 5 mice from the group with the highest tolerated concentration on day 14. If no toxicity is observed at 10mg/kg, the dose is increased until the maximum tolerated dose is determined (~ 10% incidence of clear toxicity).

Prophylactic Efficacy against LT in mice (mortality endpoint)

Initially, efficacy studies involving single injections of LT and single s.c. doses of drug candidate are carried out to eliminate molecules that have insufficient efficacy for further study. For each experiment 10 mice (control group) are injected s.c. with 0.3mL saline, and 10 (treated group) s.c. with drug candidate in 0.3mL saline, to be administered 5 minutes prior to the LT injection. All 20 animals will then be injected with 50μg of PA combined with 10μg of LF (4 x LD50). Surviving mice are observed for 14 days for signs of LT-induced nonlethal toxicity.

Prophylactic Efficacy against LT in mice (MEK-1 cleavage endpoint)

Related experiments will use the ratio of LF-cleaved to uncleaved MEK-1 in macrophages as an endpoint. Because it is difficult to isolate sufficient numbers of monocytes from peripheral blood in mice, uninduced peritoneal macrophages are used. For each experiment 10 mice (control group) are injected s.c. with 0.3mL saline, and 10 (treated group) s.c. with drug candidate in 0.3mL saline, to be administered 5 minutes prior to an i.p. LT injection. At $t = 2$ hours after injection, mice are sacrificed and peritoneal macrophage isolated by flushing the peritoneal cavity with 4mL of 0.34 M sterile-filtered sucrose.. Each mouse is expected to yield roughly 3×10^6 macrophages (Lefkovits and Benvenuto, Immunological Methods, Vol II, Academic Press, New York, p.291 (1981). The suspension is immediately combined with an equal volume of 2% sodium octadecylsulfate containing 2mM EDTA and 2mM phenanthroline in order to lyse the macrophages and stop LF activity. The ratio of cleaved to uncleaved MEK-1 is determined using Western Blot analysis with a specific anti-MEK-1 monoclonal antibody. Based on the kinetics of MEK cleavage in macrophages and the rapid activity

of lethal toxin rodents, 2 hours of exposure to LT should be sufficient time for measurable MEK cleavage to occur in macrophages (Tonello *et al.*, *Nature* **418**:386 (2002), Fish *et al.*, *J. Infect. Dis.* **118**:114-124 (1968); Welkos *et al.*, (1986)). Western Blot analysis should be sufficient for determining the cleaved to uncleaved MEK-1 ratio because it has been used to determine inhibition of LF activity inside live culture macrophage cell lines (Tonello *et al.*, 2002), and 20ng of MEK-1 (cleaved + uncleaved) has been found more than sufficient for quantitation using this technique.

Usually, drug candidates that cause > 4-fold increase in lifespan relative to controls and a statistically significant (P>0.95) decrease in the cleaved/ uncleaved MEK-1 ratio in the prophylactic studies are carried forward. Approximately 12 such molecules are chosen for further studies.

Efficacy against LT in mice at t = 1 hour post injection:

The 12 candidates that meet criteria outlined in the prophylaxis study with MEK-1 cleavage endpoint will undergo a study in mice in which the candidates are administered at **t** = 1 hour, 2 hours, and 3 hours after injection using 10 control mice and 20 treated mice. (Sets of 20 treated mice are used for each candidate and dose schedule to allow statistical significance even if 50% of the treated mice die before the **t** = 3 hour injection time). Surviving mice from treated groups are observed for 14 days after the first injection of LF + PA.

Only drug candidates that increase lifespan at least 2-fold relative to controls (with statistical significance, P> 0.95) are considered for the oral prophylaxis studies. However, compounds with very strong prophylactic activity will also be included for further study in the live challenge experiments, even if they are not very active in the therapeutic efficacy study, because the LT concentrations are well below 4 x LD_{50} until a very late stage of the infection. Approximately 8 compounds are chosen for the next step.

[redacted]

Oral prophylaxis in LT-treated mice:

Compounds active in the mortality endpoint prophylaxis study with adequate PK are tested for oral prophylaxis in mice. "Adequate oral PK" is defined as >40% oral bioavailability and a serum half-life > 2.5 hours. Both oral and s.c. PK are determined for the compounds on a contract basis by Cerep, Inc., enabling calculation of the serum half-life and % oral bioavailability.

Approximately 6 compounds are chosen for oral activity studies in mice, based on their optimal performance in the oral PK studies. The procedure for these studies is very similar to the s.c. studies in the prophylaxis study with a mortality endpoint: for each experiment 10 mice (control group) are treated p.o. with 0.1mL vehicle, and 10 mice (treated group) s.c. with a selected drug candidate in 0.1mL vehicle, to be administered t minutes prior to the LT injection. The value of t will be such that the time between agent administration (oral gavage) and LT injection will be longer so that the mean peak concentration of agent in plasma corresponds with the time of LT injection (based on the oral PK data). All 20 animals will then be injected with 50μg of PA combined with 10μg of LF (4 x LD_{50}). Surviving mice are observed for 14 days for signs of LT-induced nonlethal toxicity.

Long-term non-GLP toxicity studies:

Six of the most promising candidates, chosen based on the mouse and rat efficacy, acute toxicology and PK studies, will undergo extensive, long-term, non-GLP toxicity studies in mice, with complete blood workup and postmortem organ histopathology based on a multiple s.c. injection schedule (2x daily for 5 days) suitable for live bacillus anthracis experiments. To the extent possible, the candidate compounds are chosen to represent a variety of structural subclasses. The maximum tolerated dose at this schedule will be determined in this way.

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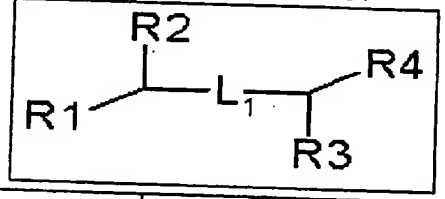
Structures and Activities for inhibitors of Anthrax Lethal Factor (LF) and Matrix Metalloproteinase 1 (MMP1).

Generic Structure:



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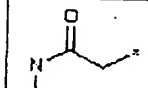
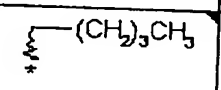
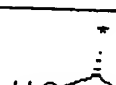
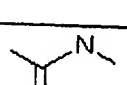
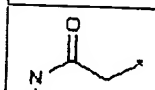
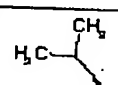
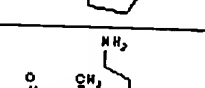
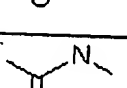
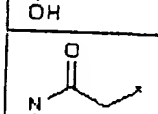
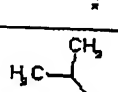
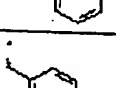
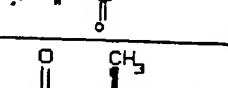
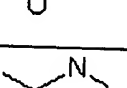
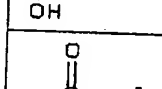
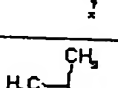
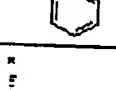
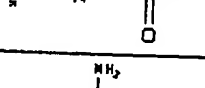
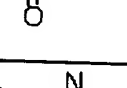
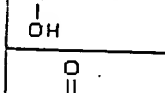
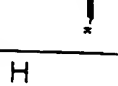
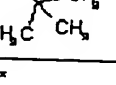
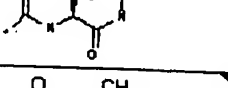
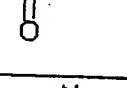
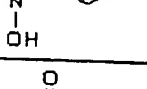
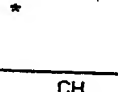
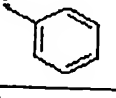
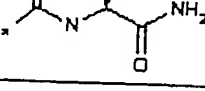
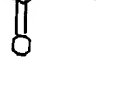
					<200	<1
					<30	<1
					<30	<1
					<200	<1
					>>30	>>100
					<30	<1

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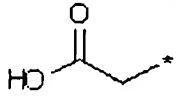
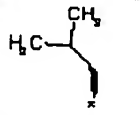
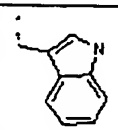
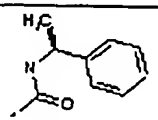
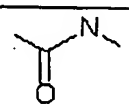
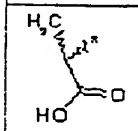
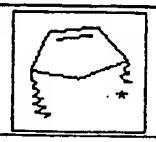
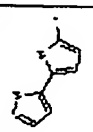
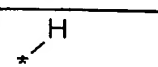
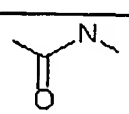
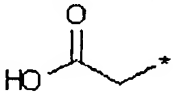
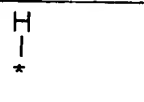
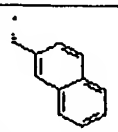
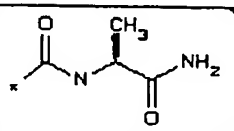
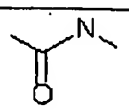
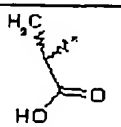
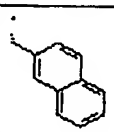
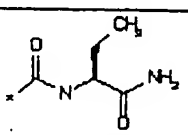
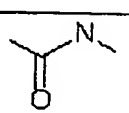
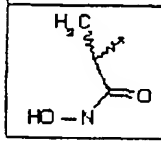
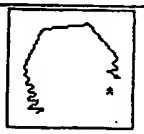
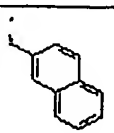
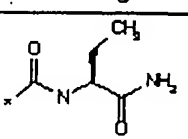
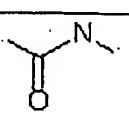
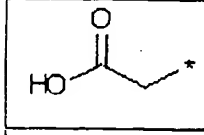
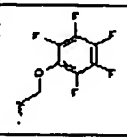
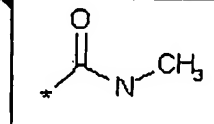
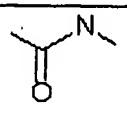
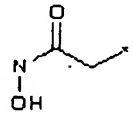
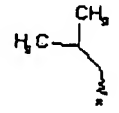
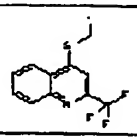
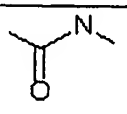
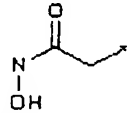
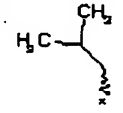
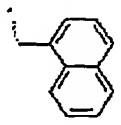
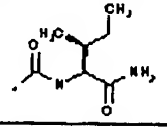
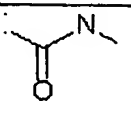
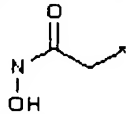
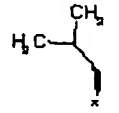
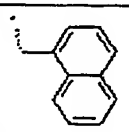
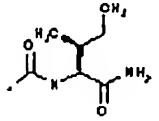
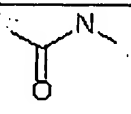
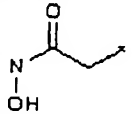
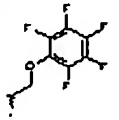
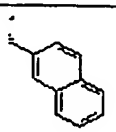
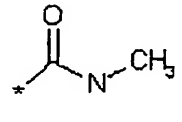
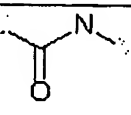
					>30	<1
					>30	
					>>30	>>30
					>>30	
					>>30	

R1	R2	R3	R4	L1	IC50 LF micromolar (No BSA)	IC50 MMP1 micromolar (+ BSA)
					>>30	
					>>30	<100
					<30	<1
					<30	<1
					>>30	>>100

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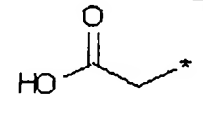
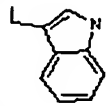
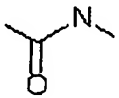
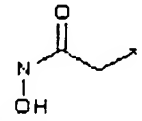
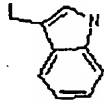
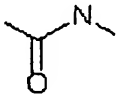
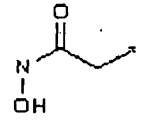
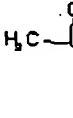
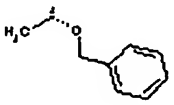
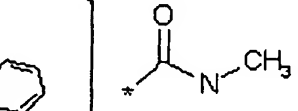
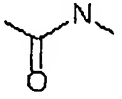
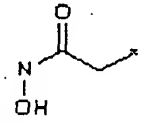
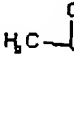
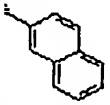
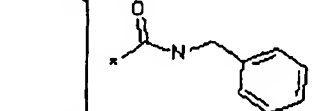
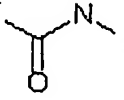
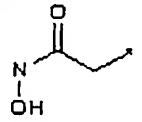
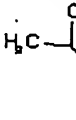
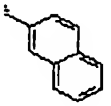
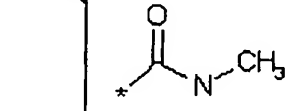
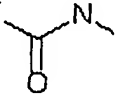
					>>100	
					<200	45
					>>100	
					<30	<1
					<200	<1

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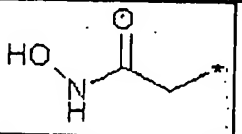
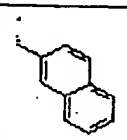
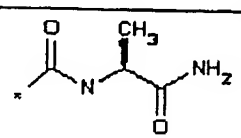
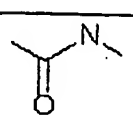
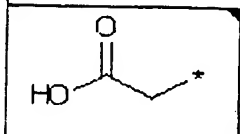
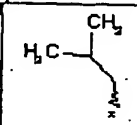
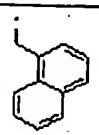
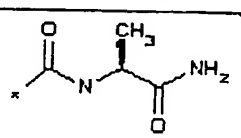
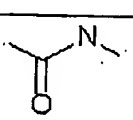
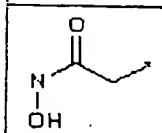
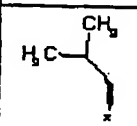
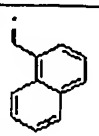
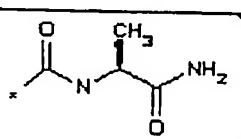
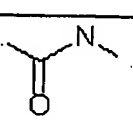
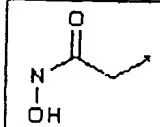
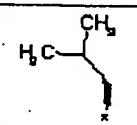
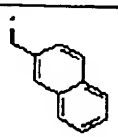
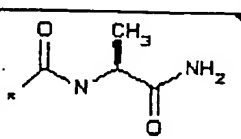
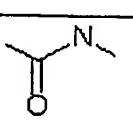
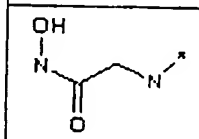
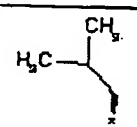
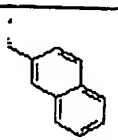
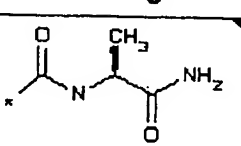
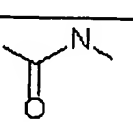
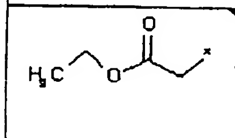
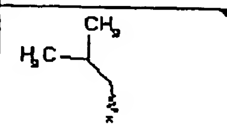
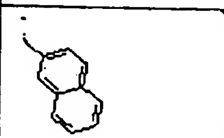
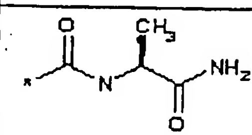
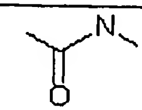
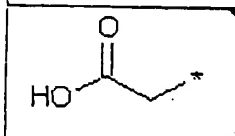
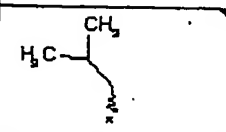
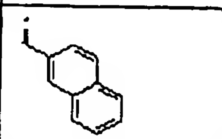
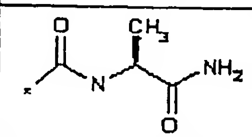
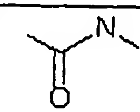
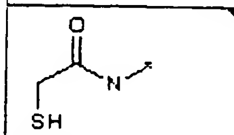
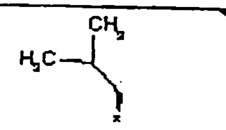
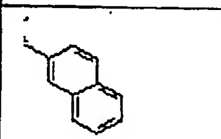
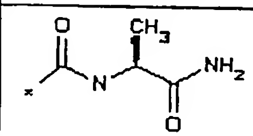
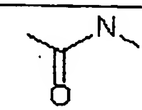
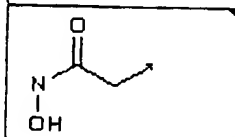
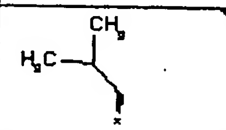
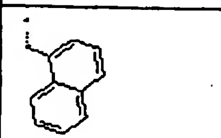
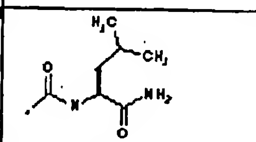
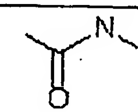
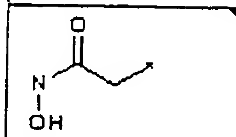
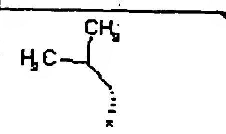
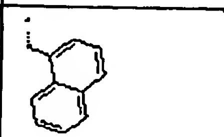
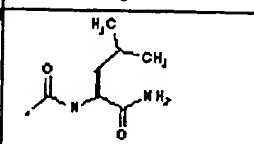
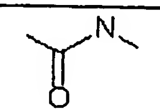
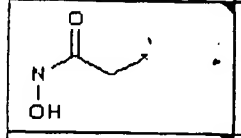
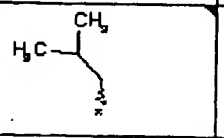
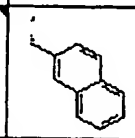
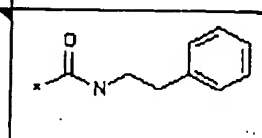
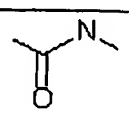
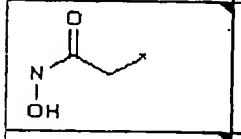
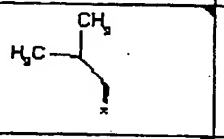
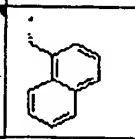
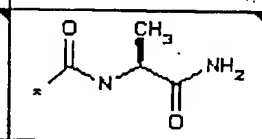
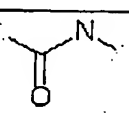
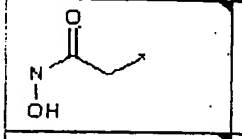
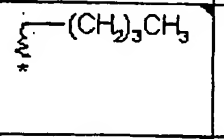
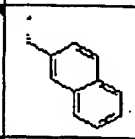
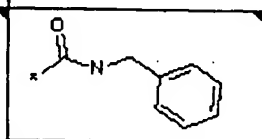
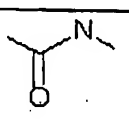
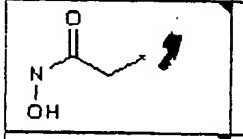
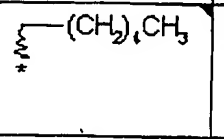
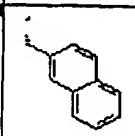
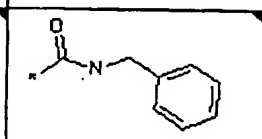
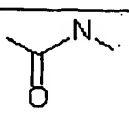
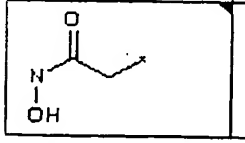
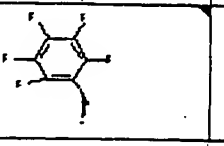
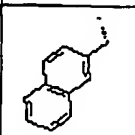
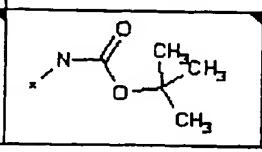
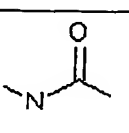
					>>100	
					>>30	
					<200	<100
					>>100	<1
					>>100	>>100

R1	R2	R3	R4	L1	IC50 LF micromolar (No BSA)	IC50 MMP1 micromolar (+ BSA)
					>>100	>>100
					>>30	
					<200	<1
					<30	<1
					<200	

R1	R2	R3	R4	L1	IC50 LF micromolar (No BSA)	IC50 MMP1 micromolar (+ BSA)
					<30	<1
					<30	<1
					30	<1
					>100	<1
					>100	>>100

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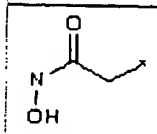
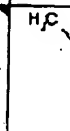
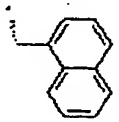
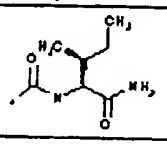
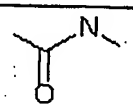
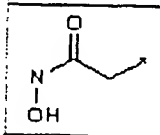
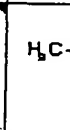
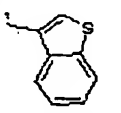
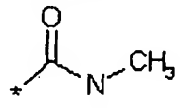
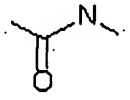
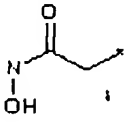
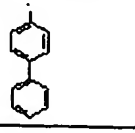
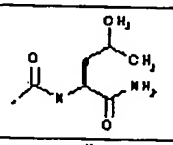
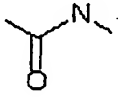
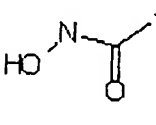
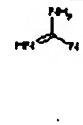
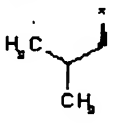
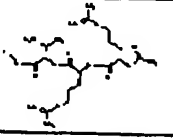
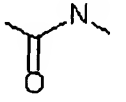
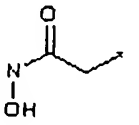
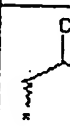
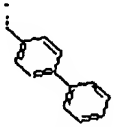
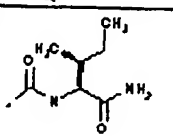
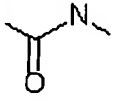
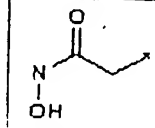
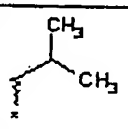
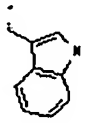
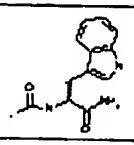
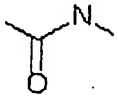
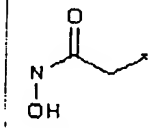
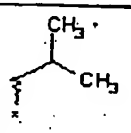
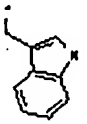
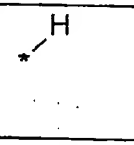
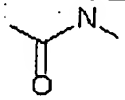
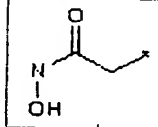
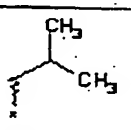
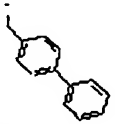
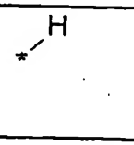
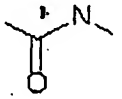
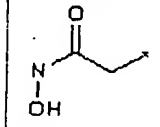
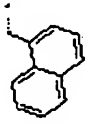
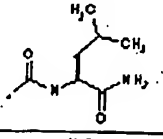
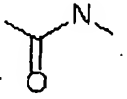
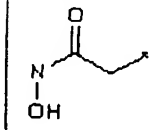
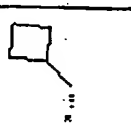
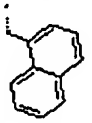
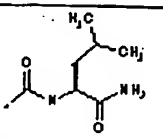
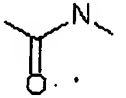
					<30	<1
					<30	
					<30	<100
					<30	<100
					<30	<1

R1	R2	R3	R4	L1	IC50 LF micromolar (No BSA)	IC50 MMP1 micromolar (+ BSA)
					<30	<1
					<30	<100
					<30	<100
					<30	<1
					<30	<1

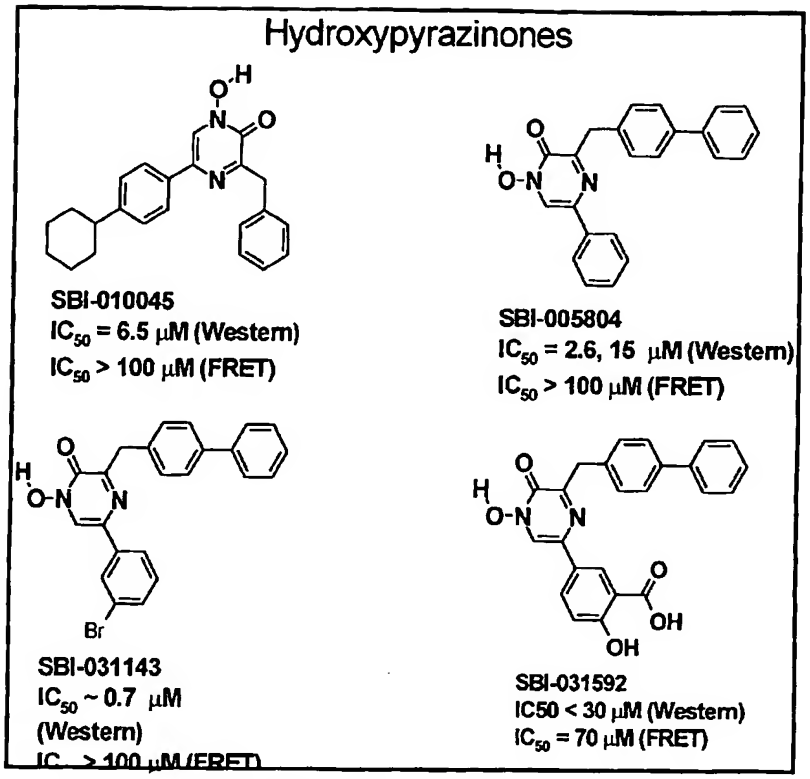


Figure C-3. Comparison of Western Blot and FRET assay results for hydroxypyrazinones leads

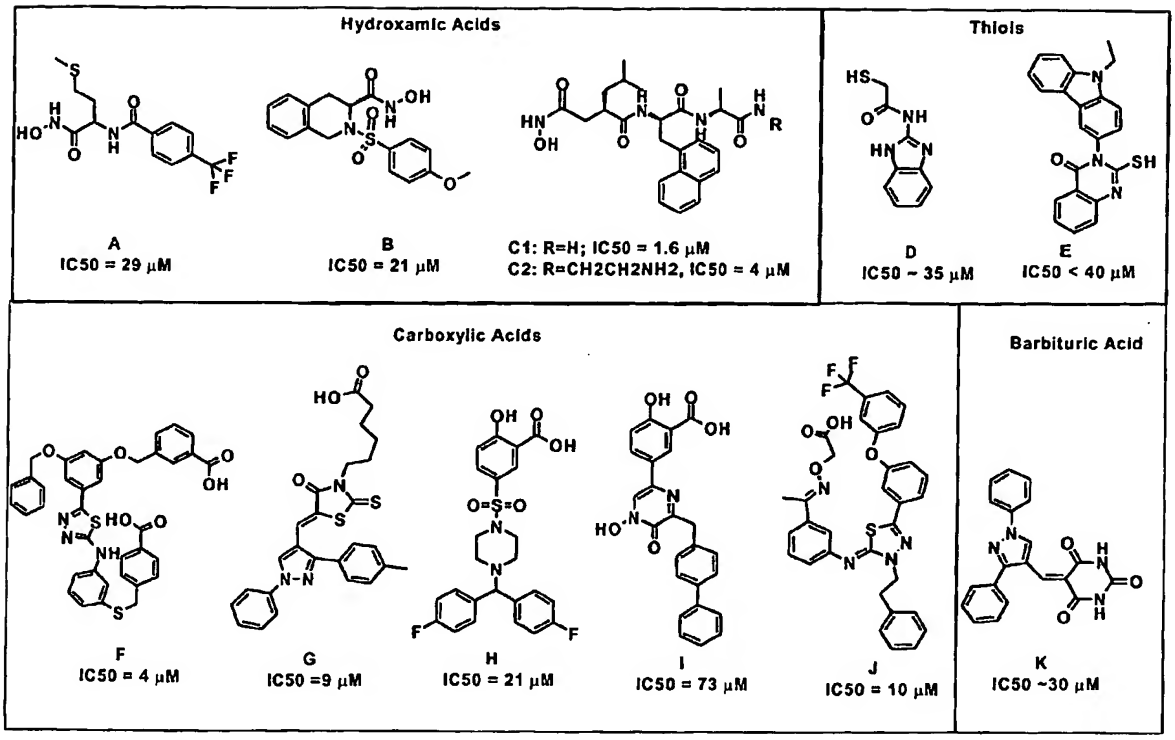


Figure C-4. LF inhibitor scaffolds and their activities in the FRET assay.

SAR for Hydroxamic Acid LF Inhibitors From Phase II
IC_{50} values in μM, FRET assay

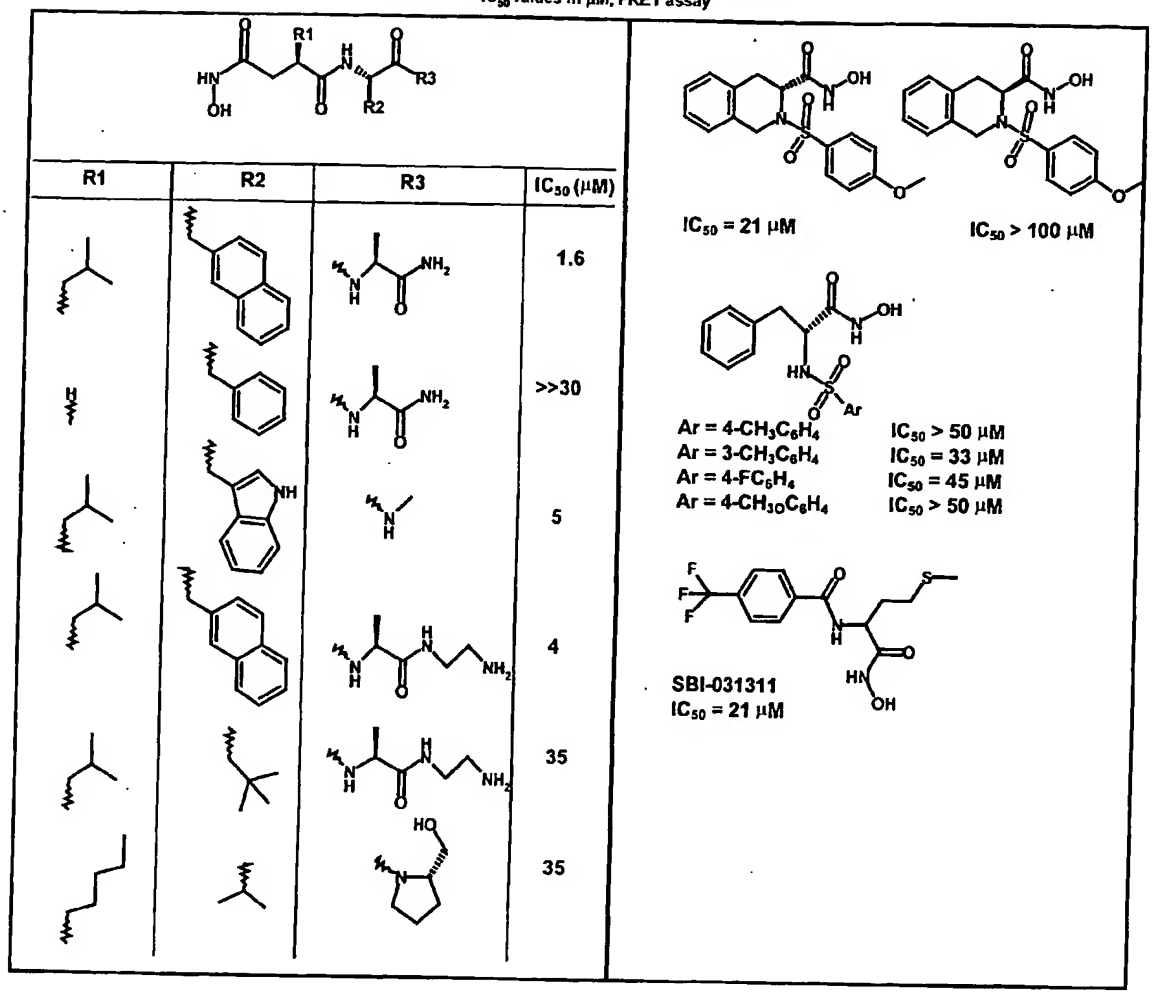


Figure C-5. Hydroxamic acid derivative structures and activities

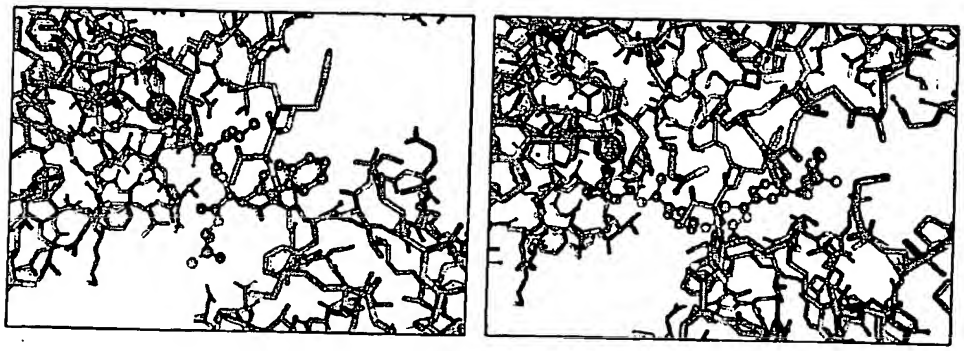


Figure C-6. Use of LF – inhibitor docking models. Models of candidate inhibitor scaffolds C (left) and J (right) docked with LF.

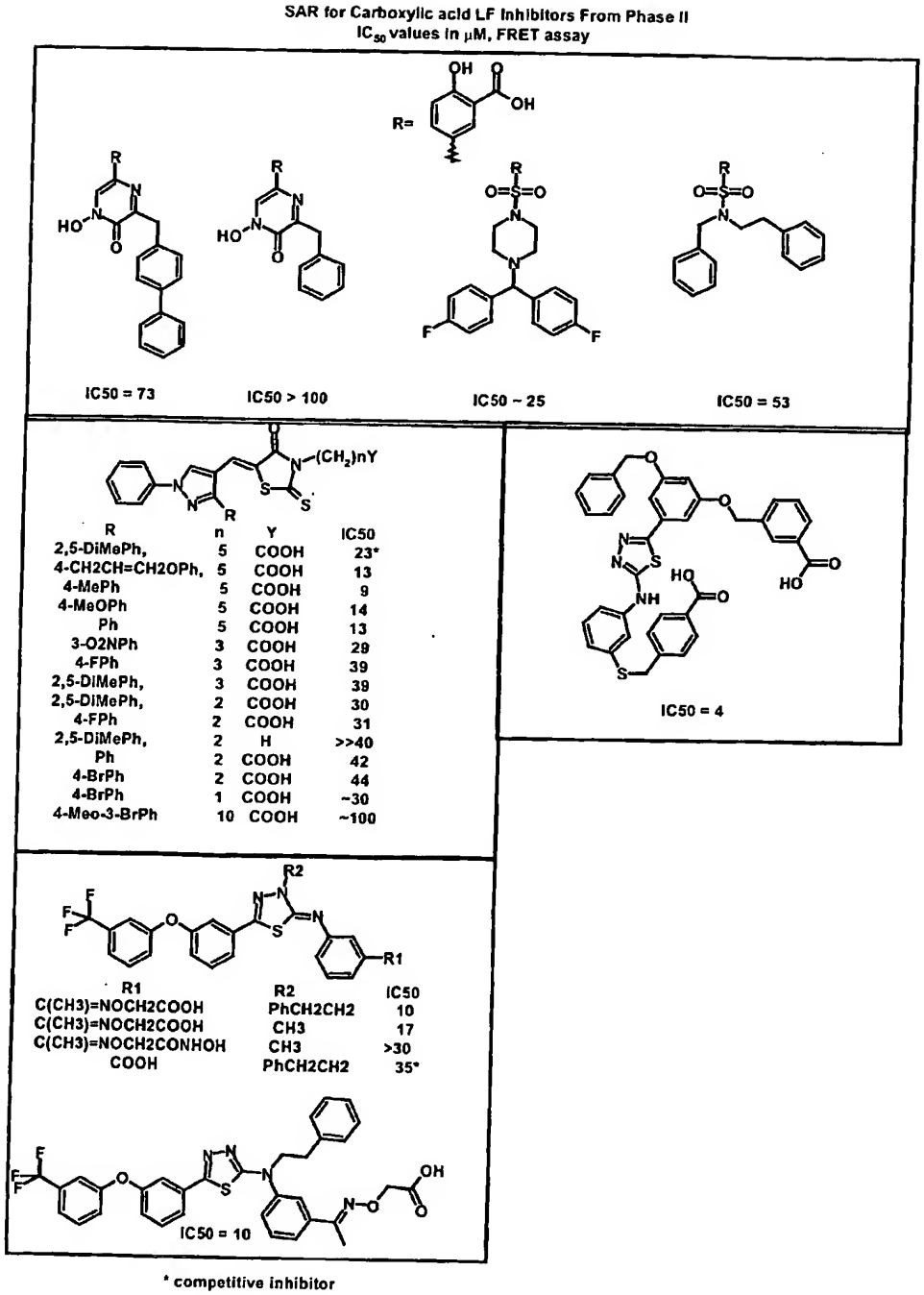


Figure C-7. Carboxylic acid derivative structures and activities

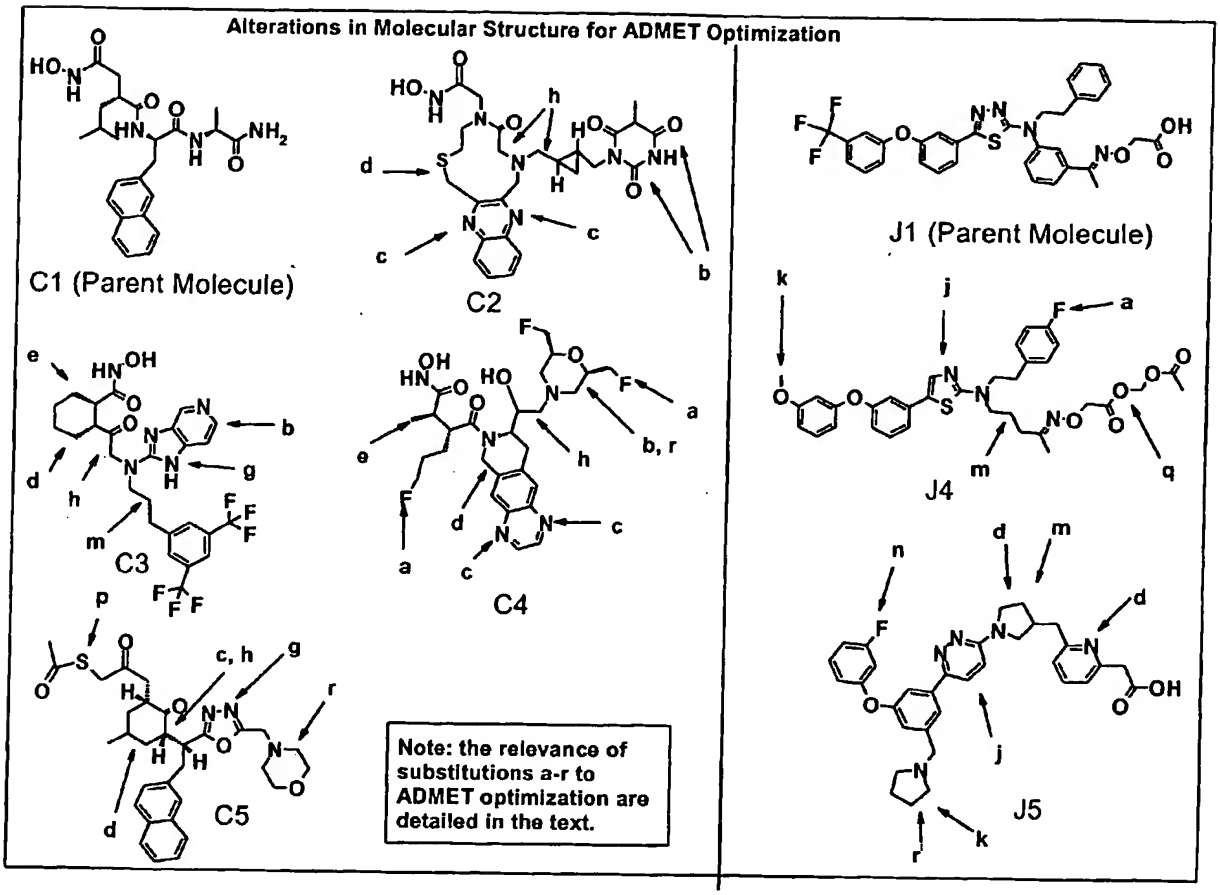


Figure D-4. Structural alteration schemes for ADMET optimization.

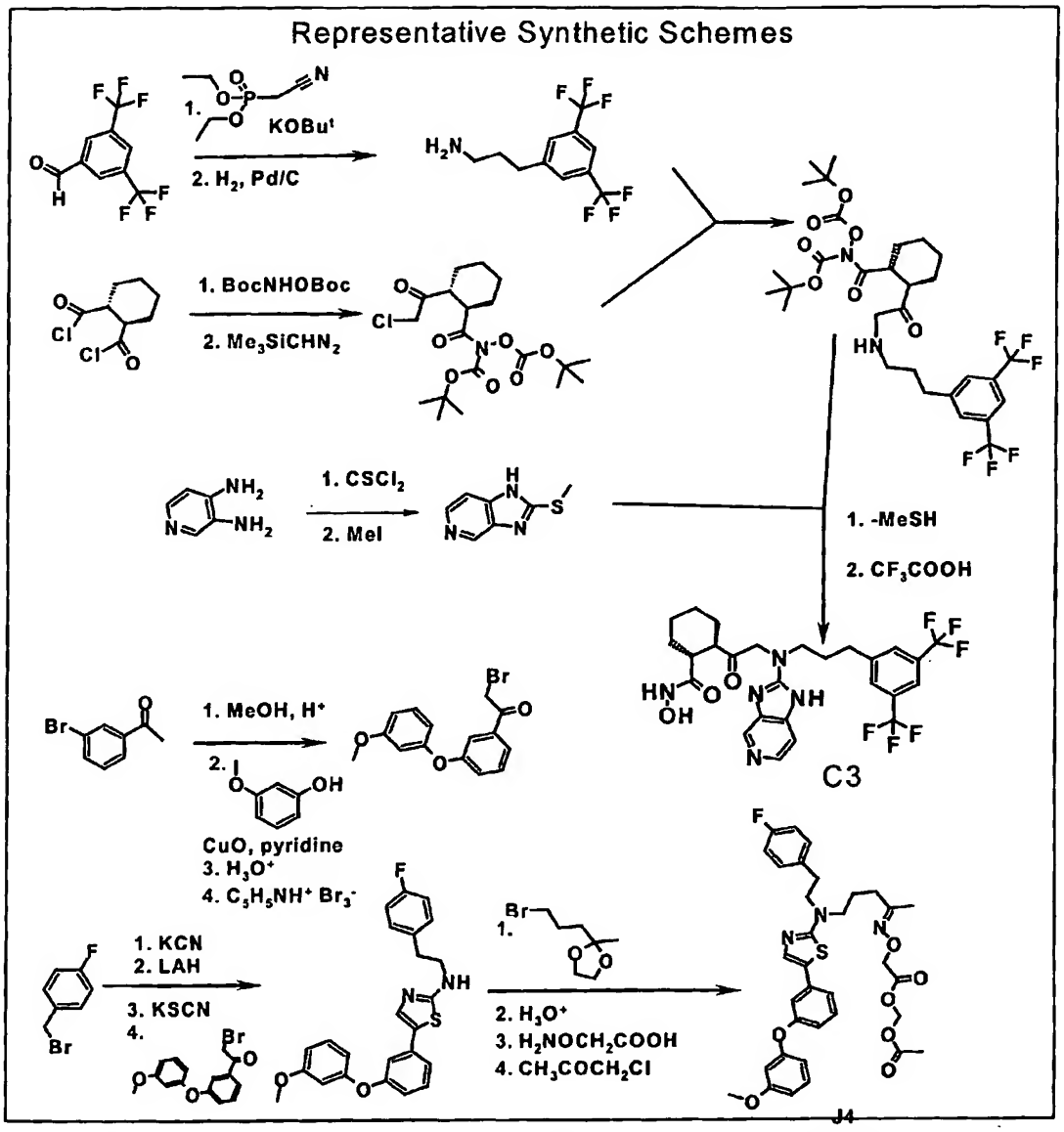


Figure D-5. Representative synthetic schemes for ADMET optimization alterations shown in Figure D-4

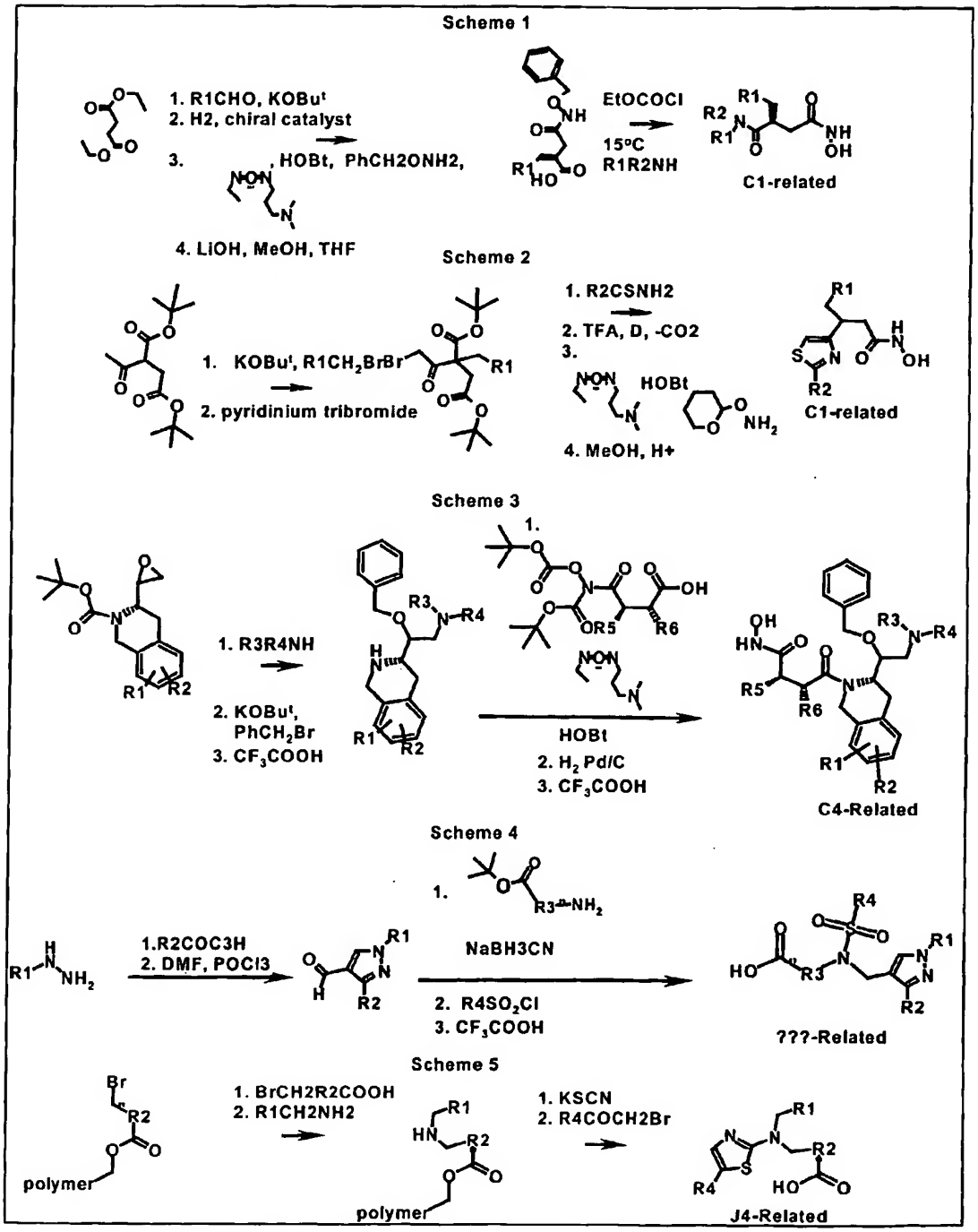


Figure D-6. Synthetic schemes for combinatorial libraries.

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